ORIGINAL ARTICLE

Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries

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Background: The aetiology of atypical haemolytic uraemic syndrome (aHUS) is, in contrast to classical, Shiga-like toxin induced HUS in children, largely unknown. Deficiency of human complement factor H and familial occurrence led to identification of the factor H gene (*FH1*) as the susceptibility gene, but the frequency and relevance of *FH1* mutations are unknown.
Methods: We established a German registry for aHUS and analysed in all patients and 100 controls the complete *FH1* gene by single strand confirmational polymorphism and DNA sequencing. In addition, complement C3 and factor H serum levels were assayed. Demographic data at onset of aHUS and follow up were compared for the mutation positive and negative groups.
Results: Of 111 patients with aHUS (68 female, 43 male, mean age 33 years) 14% had *FH1* germline mutations, including two of eight patients with familial aHUS.For each of these eight patients, both parents were tested, and we were able to trace the mutation for five cases. In the other three cases (one with the mutation 3749 C/T, one with 3200 T/C, and one with 3566+1 G/A), we could not detect the mutation in either parent, although paternity was proven by genetic fingerprinting, suggesting that these subjects have new mutations. C3 was decreased in five mutation carriers but also in two non-carriers, and factor H was decreased in none of the carriers, but elevated in six carriers and 15 non-carriers. Clinical parameters including associated medications and diseases, and outcome of aHUS and of post-aHUS kidney transplantation were similar in the mutation positive and negative groups.
Conclusion: *FH1* germline mutations occur with considerable frequency in patients with aHUS. Hypocomplementaemia is not regularly associated with a germline mutation, and factor H serum levels can even be elevated. Screening for *FH1* mutations contributes to the classification of aHUS.

Haemolytic uraemic syndrome (HUS) is a severe disease frequently leading to end stage renal failure.[1] Clinical features include rapid deterioration of renal function, anaemia negative for Coombs' test, low platelet count, elevation of lactate dehydrogenase, decreased haptoglobin, and occurrence of fragmented red cells in the blood smear.

Current classification of HUS delineates two major types.[2] Classical HUS occurs almost exclusively in childhood and is caused by bacteria releasing Shiga-like toxins. The disease starts with signs of enteritis, generally initiated by *Escherichia coli* strains, mainly O157.[3] Atypical HUS (aHUS) usually occurs in adults, and has been reported in association with a variety of conditions such as therapeutic drug usage (ovulation inhibitors, immunosuppressive agents), diseases (malignancies, systemic lupus erythematosus) or pregnancy, and after childbirth.[4] Differentiation of classical and aHUS is important for both treatment and outcome, as patients with aHUS require plasmapheresis with replacement by fresh frozen plasma.[5]

Familial occurrence of aHUS is reported in siblings,[6–12] in a few families with autosomal dominant inheritance[13–16] and rarely with autosomal recessive transmission.[17] In some families, affected individuals exhibit decreased plasma levels of C3,[12 18] indicating defective complement control, and suggesting a role of complement regulators for the disease process.[19 20]

Factor H is a central fluid phase regulator of complement, and both deficiency and functional inactivation of the plasma protein results in uncontrolled complement activation.[21] The human factor H gene (*FH1*) is located on the short arm of chromosome 1 (1q32),[22] a region that includes the regulators of complement activation gene cluster. Linkage analysis indicated that *FH1*, which is located in this cluster, could be a candidate gene for aHUS.[16] Subsequently, independent groups have reported germline mutations in *FH1* in additional families, thus corroborating its pathogenetic relevance.[23 24]

In order to identify the frequency of *FH1* mutations in aHUS in general, we established a large registry for aHUS, identified mutations in patients with and without a family history, and compared clinical features of mutation positive and negative patients.

METHODS

Registry for aHUS

Nine hundred nephrologists in Germany, Austria, northern and central Switzerland, and northern Italy were contacted in 1998, 2000, and 2002 in order to establish a registry for aHUS in German-speaking countries. Included in this study were all patients for whom blood for further analyses was available. Criteria for HUS were rapid deterioration of renal function, non-immunogenic anaemia, low platelet count and haemolysis, for example, elevated lactate dehydrogenase, decreased haptoglobin, occurrence of fragmentocytes in peripheral blood smear, and thrombotic microangiopathy in renal biopsy. All cases were reevaluated for aHUS by tests for antibodies for the following *E coli* MTP strains: O157, O26,

Abbreviations: aHUS, atypical haemolytic uraemic syndrome; HUS, haemolytic uraemic syndrome

Table 1 Demographic data of 98 patients with atypical haemolytic uraemic syndrome

	Mutation positive	Mutation negative	Total	p Value
Patients (n)	16	95	111	—
Age at onset of HUS (median)	1–59 (29)	2–78 (32)	1–78 (32)	0.074
Sex (M/F)	7/9	36/59	43/68	0.428
Complement activation parameters				
Decreased /(elevated) serum C3	5 / (1)	2 / (14)	7 / (15)	—
Decreased /(elevated) factor H	0 / (6)	0 / (15)	0 / (21)	—
Associated conditions				
Familial aHUS	2	6	8	0.342
Drugs (ovulation inhibitors)	3 (3)	14 (6)	17 (9)	0.459 (0.120)
Gestation/after childbirth	1	14	15	0.322
Malignant tumours	0	6	6	0.384
Signs of infections	2	11	13	0.593
Other conditions (SLE etc).	4	8	12	0.070
Treatment and outcome				
Plasma exchange (FFP alone)	7 (1)	69 (2)	76 (3)	0.025 (0.376)
Relapse in genuine kidneys	5	23	28	0.374
Chronic renal failure	1	15	16	0.284
End stage renal failure	13	69	82	0.350

p Value indicates statistical differences of mutation-positive *v* mutation-negative patients.
FFP, fresh frozen plasma; SLE, systemic lupus erymathosus.

O103, O111, O3, O8, O91, and O145. Complement C3 was measured in serum samples by standard nephelometry (Dade-Behring, Marburg, Germany).

Demographic features included associated conditions, diseases, drugs and other toxins, family history, laboratory data at presentation, and modalities of treatment. Follow up data were reassessed in October 2002 by a detailed questionnaire regarding outcome of renal function and possible kidney transplantation. Relapse of aHUS not causing complete loss of graft function, and organ loss due to non-surgical reasons were analysed. Deterioration of renal function was defined as serum creatinine levels above 1.5 mg/dl.

Factor H gene mutations

Analysis of the *FH1* gene was performed using DNA extracted from 10 ml peripheral EDTA blood by standard procedures (Qiagen Kit; Qiagen, Germany). All 23 exons except exon 10, which encodes an exon specific to the alternatively spliced product FHL-1,[21] were screened for mutations by single strand conformation polymorphism (SSCP) of PCR products. We used standard primers as reported.[24] Novel primer pairs were designed for exons 9 and 21 in order to avoid amplification of homologous exons in the factor H related genes *FHR1–FHR5*. The new primers were: for exon 9, CTCATTTACTTTATTTATTTATCATTGT (forward primer), TGAACATGCTAGGATTTCAGAGTA (reverse primer); and for exon 21, GAAATATATTTGTAACTGTTATC (forward primer), and GTTTTTCAGGTTCCAACTCTC (reverse primer). When SSCP revealed an abnormal band pattern, the corresponding exon was sequenced. Once a mutation was identified, we invited the carrier's parents and the relatives who had been affected by aHUS for genetic testing. For controls, we used blood samples of 100 healthy German blood donors from the University of Freiburg. Nucleotide and amino acid numbering was used according to the published cDNA sequence.[25] Structural composition of the factor H protein, arranged as 20 tandem short consensus repeats (SCRs), was also noted.

Prediction of effects of amino acid substitutions

The PolyPhen server (http://www.bork.embl-heidelberg.de/PolyPhen/)[26] was used to predict the likely impact of non-synonymous amino acid substitutions observed in patients and controls.

Factor H ELISA

Factor H concentrations were determined in serum using an ELISA assay.[27] The protocol of this study was approved by the ethics committee of the University of Freiburg. All subjects gave informed consent.

For statistical analyses we used Fisher's two-tailed exact test to compare small groups. For larger groups, the unpaired two-sided χ^2 test was used. $P<0.05$ was taken as statistical significance.

RESULTS

Registry

The registry of aHUS for German speaking countries included (as of June 2003) 111 patients with aHUS of non-transplanted kidneys. One case was excluded from the study because of elevated MTP-O157 antibodies. The demographic data are presented in table 1. There were 68 females and 43 males. Age at diagnosis of aHUS was 1 to 78 years (median 32 years); the series included four children aged 1, 2, 2, and 6 years, three adolescents of 13, 14, and16 years, and four of 17 years. In 81% of the patients aHUS occurred in the decade from 1993 until 2002.

DNA variants of the factor H gene

Analyses of the 111 patients with aHUS revealed 17 germline DNA variants in the *FH1* gene (table 2). These variants did not occur in the control group (table 3). Consequently, these findings were interpreted as germline mutations. Mutations were observed in 16 patients, and one patient had two mutations. The mutations are located in exons 14, 15, 17, and 19–23, representing at the protein level SCRs 11, 12, 14, and 16–20, respectively. Mutations clustered in the C-terminus of factor H; 82% of the mutations were located in regions coding for SCRs 16 to 20 (exons 19–23). The majority of mutations (13 of 17) were missense, resulting in single amino acid exchanges. Of the additional mutations, one caused a frameshift, one introduced a premature stop at codon 714, one mutation caused a single amino acid deletion at codon 1216, and one mutation affected splicing at nucleotide 3566 (exon 22, SCR 19). All mutations analysed are heterozygous, indicating that each of the individual patients had one intact and one defective allele. All but one of the mutations occurred only once in this series.

For nine of 17 mutation carriers, both parents were tested. Three patients had new (de novo) mutations. The patient

Table 2 Novel factor H gene mutations identified in the new registry

Case no/ age	Mutation (cDNA nucleotide)*	Exon	Consequence (amino acid)*	SCR	C3 (0.65–1.85 g/l)**	Factor H (235–810 mg/l)**	Family
Cases with one mutation							
1/31	1963 T/G***	14	630 Cys→Trp	11	0.876	774	Sporadic. Father carrier
2/25	2214 C/G***	15	714 Ser→Stop	12	0.562	619	Sporadic. Mother carrier
3/24	2621 G/A***	17	850 Glu→Lys	14	2.0	927	Sporadic
4/22	3007 G/T***	19	978 Trp→Cys	16	0.754	703	Sibling HUS. Mother and grandmother carriers
5/33	3200 T/C***	20	1043 Cys→Arg	17	0.748	560	Sporadic
6/26	3299 C/G	21	1076 Gln→Glu	18	1.82	511	Sporadic. Father carrier
7/2	3474 T/G***	22	1134 Val to Gly	19	0.835	835	Sporadic. Mother carrier
8/37	3497 T/G***	22	1142 Tyr to asp	19	0.425	1007	Sporadic
9/28	3542 T/C***	22	1157 TrP→Arg	19	0.508	894	Sporadic
10/18	3566+1 G/A***	22	Splice defect	19	0.729	826	Sporadic
11/1	3620 C/T	23	1183 Trp→ Arg	20	0.890	619	Familial. Paternal cousin and second-degree aunt on dialysis
12/13	3701 C/T	23	1210 Arg→Cys	20	1,250	580	Sporadic
13/40	3719 delACA***	23	1216 del Thr	20	0.368	534	Sporadic
14/33	3749 C/T***	23	1226 Pro→Ser	20	1.020	1300	Sporadic
15/59	3768–71 delAGAA***	23	Frameshift	20	0.540	503	Sporadic
Case with two mutations							
16/39	3135 A/T*** and 3701 C/T	20 23	1021 Lyr→Phe 1210 Arg→Cys	17 20	0.749	787	Sporadic. Carriers: father, 3135A/T mother 3701C/T

*Numbering according to[25]; **normal range for C3 and Factor H serum concentrations (mean plus/minus 2SD of the control series of 100 probands); ***the mutation is novel.

Table 3 DNA variants of the factor H gene occurring in controls and patients of this series

Polymorphism (cDNA nucleotide)	Exon	Consequence (amino acid)	SCR	Healthy probands (n = 100)	Unrelated HUS cases	
					(Mutation+)(n = 16)	(Mutation−)(n = 95)
257 G/A**	2	62 Val→ Ile	1	0*/4**	0*/0**	0*/5**
994 C*/A/**	7	307 Ala→Ala	5	0*/0**	5*/1**	43*/5**
1277 C*/T**	9	402 His→Tyr	7	49*/39**	5*/21**	44*/46**
1492 G*/A**	11	473 Ala→Ala	8	0*/0**	5*/1**	44*/13**
2089 A*/G**	14	672 Gln→Gln	11	19*/2**	10*/4**	38*/11**
2707 C/T*	18	878 His→ His	15	0*/0**	0*/0**	1*/0**
2881 G*/T**	19	936 Glu→Asp	16	17*/3**	20*/5**	39*/12**
2923 G/T*	19	950 Gln→His	16	2*/0**	0*/0**	2*/0**
3211 C/T*	21	1046 Thr→Thr	18	0*/0**	0*/0**	2*/0**
3221 A/T*	21	1050 Asn→Tyr	18	6*/0**	0*/0**	2*/0**

*Heterozygous; **homozygous; ‡Polyphen prediction of effect of amino acid substitution.[26]
The variant *FH1* c. 2923 G/T has not been reported as a polymorphism in other series so far and may therefore be associated with a risk for HUS.

with two mutations (case 16) inherited the 3135 A/T mutation from the father and the 3701 C/T mutation from the mother. The G3007T mutation (case 4) was transmitted from the mother and the grandmother. An additional two cases inherited the mutation from the father, and two other cases from the mother (table 2). None of these ancestors had aHUS and none had impairment of renal function. Genetic testing of the sister of the patient with the 3007 G/T mutation (case 4), who also had aHUS, revealed the same mutation.

The *FH1* gene analyses of the 100 healthy controls revealed ten DNA sequence variants. Five variants (994 C/A, 1492 G/A, 2089 A/G, 2707 C/T, 3211 C/T) have no consequence at the amino acid level (silent mutations) and were therefore interpreted as polymorphisms. Four additional DNA sequence variants, which cause an amino acid exchange (62 Val→Ile, 402 His→Tyr, 936 Glu→Asp, 1050 Asn→Tyr), have been reported previously as polymorphisms.[24 28] One novel DNA variant, which also results in an exchange of a single amino acid (950 Gln→His) was found in two controls and three patients of this series (table 3).

Predicted effects of amino acid substitutions

PolyPhen is an automated tool for prediction of the likely impact of an amino acid substitution on protein structure and function.[26] Mutations observed in controls (table 3) were all predicted to be ''benign'' or only ''possibly damaging'', whereas the majority of substitutions in patients were classified as ''probably damaging'' (table 2).

Table 4 Kidney transplantations in 36 patients of this series with atypical HUS and non-surgical complications

	Mutation positive*	Mutation negative	Total	p Value
Patients (n)	6	29	35	
Organs	10	37	47	
Living donors	1	8	9	0.375
Lost organs in first year after transplantation	8	20	28	0.409
Relapse and acute rejections	8	19	27	0.332

Table 5 Reported factor H gene mutations; summary of literature reports

Case	Mutation (cDNA nucleotide)	Exon	Consequence (amino acid)	SCR	C3	Factor H	Family	Reference
Cases with one mutation								
I	155–158 del 4 bp	2	Premature Stop	1	Low	Low	Sporadic	[16] Case
II	1494 del A	11	Frameshift	8	Low	Normal	2 patients and 1 unaffected brother	[23] Fam 3
III	2940 C/T	19	956 Thr→Met	16	Normal	High	Mother carrier without HUS	[24] HUS 12
IV	3429 A/G	22	1119 Asp→Gly	19	Normal	Normal	Sibling HUS	[28] Case 2
V	3620 T/C	23	1183 Trp→Arg	20	Normal	Normal	Brother with aHUS	[35] Case rep
VI	3621 G/T	23	1183 Trp→Leu	20	Low	High	n.r.	[24,35] HUS 2
VII	3624 C/G	23	1184 Thr→Arg	20	Low	Normal	Sporadic	[28] Case 4
VIII	3639 T/G	23	1189 Leu→ Arg	20	Normal	High	Father mutation negative	[24] HUS 11
IX	3654 G/A	23	1194 Gly→Asp	20	n.r.	n.r.	n.r.	[36]
X	3663 T/C	23	1197 Val→Ala	20	Low	Low	Sporadic	[23] nap
XI	3701 C/T	23	1210 Arg→Cys	20	Normal.	Normal	Father and 2/5 siblings carriers, no HUS	[23] R 16
XII	3701 C/T	23	1210 Arg→Cys	20	Low	Normal/High	Siblings with HUS, father carrier	[23] Fam 24
XIII	3701 C/T	23	1210 Arg→Cys	20	Low.	Normal	5 other (healthy) carriers including sister and father	[29]
XIV	3716 C/G	23	1215 Arg to Gly	20	Normal	Normal	Sporadic	[6,28] Case 3
XV	3717 C/A	23	1215 Arg→Gln	20	Low	Normal	Carriers: father and grandfather	[23] Fam 1
XVI	del 24 bp*	23	Premature Stop	20	Low	Low	Autosomal recessive	[23] Fam 29
Cases with two mutations								
XVII	3299 C/G and	21	1076 Gln→Glu	18	n.r.	n.r.	Sporadic	[28] Case 1
	3559 del A	22	Frame shift	19				
XVIII	3645 C/T** and	23	1191 Ser→Leu	20	n.r.	n.r.	Mother HUS	[28] Case 5
	3663 T/C**	23	1197 Val→Ala	20				
XIX	3663 T/C* and	23	1197 Val→Ala	20	Low	Low	n.r.	[24] HUS 3
Partial deletion***								

*Homozygous; **mutations on the same allele; ***other chromosome, n.r., not reported; nap, note added in proof of this publication.

C3 and factor H serum levels

Serum samples were obtained from 106 of the 111 patients with aHUS. C3 analyses revealed decreased levels in seven patients (five with an *FH1* mutation and two without an *FH1* mutation) and high levels in 15 patients (one with and 14 without an *FH1* mutation). Factor H measurement resulted in elevated levels in six mutation-positive and 15 mutation-negative patients, but in none of the patients with decreased levels.

Clinical findings and correlations

The clinical characteristics of the 16 mutation-positive and the 95 mutation-negative patients are presented in tables 1 and 4. Mutation-positive and mutation-negative subjects showed no difference for age or putative causative conditions. In both groups there was a tendency to female gender (56% and 62%, p = 0.428). Follow up and outcome were similar in both groups, resulting in end-stage renal failure in 81% and 73%, respectively (13/16 *v* 69/95, p = 0.35).

Six mutation-positive and 29 mutation-negative patients underwent kidney transplantation, with repeated transplantations in three and seven subjects in each group respectively (table 4). From the total of 47 transplanted organs, nine were from living donors; one in the mutation-positive group and eight in the mutation-negative group. Relapse of aHUS and acute rejections occurred in eight mutation positive and 19 mutation negative recipients. Graft loss within the first year due to non-surgical reasons occurred in eight organs (80%) of mutation positive and 20 organs (54%) of mutation negative recipients. Statistically, outcome of kidney transplantation did not differ between the two groups (table 4).

Familial aHUS was present in eight patients. In six of these patients, pedigree analysis revealed evidence for autosomal

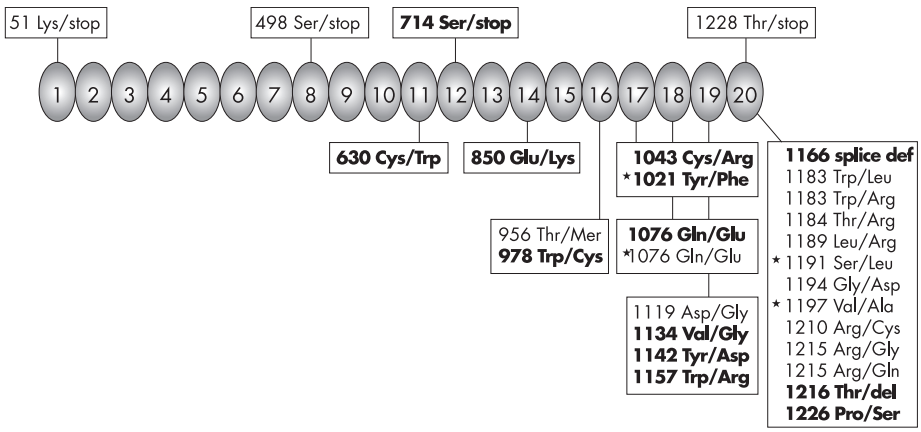

Figure 1 Factor H gene mutations associated with atypical haemolytic uraemic syndrome (aHUS). The domain structure of factor H is shown. The position of the 12 novel mutations reported in this study are shown in black and that of the mutations reported in the literature are shown in grey characters (all fused from tables 2 and 5). Double mutations are marked with*.

dominant transmission with variable penetrance, whereas two brothers in the seventh family and two sisters in the eighth were affected, leaving the mode of transmission open to debate. The latter family was the only family with an *FH1* germline mutation detected in this study (table 2). In another index case, the father was on dialysis, and was not available for genetic testing.

DISCUSSION

We established a registry of German speaking countries, comprising 111 patients from Germany, Austria, Switzerland, and southern Tyrolia/Italy for a systematic clinical and molecular genetic evaluation of patients with atypical HUS (aHUS), (HUS not induced by Shiga-like toxin).

Based on this registry, we analysed the complete *FH1* gene, a candidate susceptibility gene for aHUS,[16] and found *FH1* mutations in 13% of the patients. With this approach, we identified 17 new disease-associated *FH1* mutations. One patient had two mutations, and only one of the detected mutations occurred twice.

Thirteen of the mutations are novel, and only three mutations have been reported previously (tables 2 and 5). The identified mutations are spread over eight exons, and the reported mutations involve an additional two exons (fig 1). This distribution suggests that the entire *FH1* gene should be evaluated for genetic classification of aHUS. The relevance of our findings is sustained based on a parallel search of the *FH1* gene in blood samples of 100 controls.

Two of our 16 mutation-positive patients had a relative affected by aHUS, indicating familial cases. From the literature a minority of 25% had a family history for aHUS (table 5).[23 24 28] In most families only siblings are affected by aHUS, (twice in this series and four instances previously reported).

Tracing the mutations back to the former generation was possible in six of our patients and five reported cases (table 5), but none of the ancestors had episodes of an aHUS. This can be explained in two ways, either as autosomal-dominant transmission with reduced penetrance, or as a recessive trait. The latter has been confirmed by molecular genetic and clinical data of one reported family, (table 5).[19 23] A similar scenario is likely for our patient 13 who exhibited two mutations, one inherited from the father, the other from the mother.

Most mutations (76%) in this registry represent single amino acid changes, and the remainder introduce premature stop codons or cause a deletion of an amino acid in the factor H protein. The fraction of single amino acid mutations is similar to that reported in the literature (72%). These missense mutations may affect secretion of the protein as well as biological function. Recent analyses have begun to unravel the precise function of the C-terminus of factor H and the impact of individual mutations on protein function.[29–31] Both a heparin and a C3b binding site have been identified in SCR 20 of factor H,[32] and the other C-terminal SCRs contribute to this biological activity.

Hypocomplementaemia and factor H deficiency are considered key features of hereditary aHUS.[19 24 33] Such conditions, however, are rarely reported in cases with proven *FH1* mutations. Factor H levels may even be elevated (table 5). Data from our registry show normal C3 levels in 10/16 cases and elevated serum factor H in 6/16 cases (table 2). The frequency of high factor H levels indicates upregulation of factor H synthesis.

This registry allows, for the first time, comparisons of family history, including pedigree analysis of *FH1* mutation-positive patients with aHUS and *FH1* mutation-negative patients with aHUS (tables 2 and 4). Remarkably, we were able to detect an *FH1* mutation in only two of eight patients with familial aHUS. This result corresponds well with another study revealing a 2/19 detection rate.[28] These data suggest the existence of at least one other, so far undetected, susceptibility gene for aHUS.

Associated factors that are considered to trigger aHUS, such as exposure to drugs, the contraceptive pill, pregnancy, and malignancies or non-*E coli* induced infections, occurred with similar frequency in both groups. Again, outcome, including relapse rates in non-transplanted kidneys, did not differ statistically between the groups, with end stage renal failure in 81% of the mutation-positive and 73% in the mutation-negative patients. Thirty-six patients with aHUS underwent kidney transplantation (table 4). Of the 47 transplanted kidneys, the number of grafts lost due to non-surgical reasons within the first year was not significantly different in the mutation-positive and the mutation-negative group. These data characterise the poor prognosis of aHUS as similarly shown by others.[4 19 34]

Regarding the 87% of cases in which we have been unable to identify an *FH1* mutation, it may be speculated that other complement regulators such as C4BP, MCP, DAF, and CR1 might be involved the the disease process. In summary, this registry-based study demonstrates that germline mutations of the *FH1* gene are relatively frequent in patients with aHUS, and demonstrate a clear role of this immune regulator for the development of the disease. Further biochemical and functional studies of both normal and mutated factor H protein will provide deeper insights into the pathogenesis of aHUS.

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